

Geobacter pickeringii sp. nov., *Geobacter argillaceus* sp. nov. and *Pelosinus fermentans* gen. nov., sp. nov., isolated from subsurface kaolin lenses

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The goal of this project was to isolate representative Fe(III)-reducing bacteria from kaolin clays that may influence iron mineralogy in kaolin. Two novel dissimilatory Fe(III)-reducing bacteria, strains G12^T and G13^T, were isolated from sedimentary kaolin strata in Georgia (USA). Cells of strains G12^T and G13^T were motile, non-spore-forming regular rods, 1–2 µm long and 0.6 µm in diameter. Cells had one lateral flagellum. Phylogenetic analyses using the 16S rRNA gene sequence of the novel strains demonstrated their affiliation to the genus *Geobacter*. Strain G12^T was most closely related to *Geobacter pelophilus* (94.7%) and *Geobacter chappellei* (94.1%). Strain G13^T was most closely related to *Geobacter grbiciae* (95.3%) and *Geobacter metallireducens* (95.1%). Based on phylogenetic analyses and phenotypic differences between the novel isolates and other closely related species of the genus *Geobacter*, the isolates are proposed as representing two novel species, *Geobacter argillaceus* sp. nov. (type strain G12^T = ATCC BAA-1139^T = JCM 12999^T) and *Geobacter pickeringii* sp. nov. (type strain G13^T = ATCC BAA-1140^T = DSM 17153^T = JCM 13000^T). Another isolate, strain R7^T, was derived from a primary kaolin deposit in Russia. The cells of strain R7^T were motile, spore-forming, slightly curved rods, 0.6 × 2.0–6.0 µm in size and with up to six peritrichous flagella. Strain R7^T was capable of reducing Fe(III) only in the presence of a fermentable substrate. 16S rRNA gene sequence analysis demonstrated that this isolate is unique, showing less than 92% similarity to bacteria of the *Sporomusa–Pectinatus–Selenomonas* phyletic group, including '*Anaerospira hongkongensis*' (90.2%), *Acetonema longum* (90.6%), *Dendrosporobacter quercicolus* (90.9%) and *Anaerosinus glycerini* (91.5%). On the basis of phylogenetic analysis and physiological tests, strain R7^T is proposed to represent a novel genus and species, *Pelosinus fermentans* gen. nov., sp. nov. (type strain R7^T = DSM 17108^T = ATCC BAA-1133^T), in the *Sporomusa–Pectinatus–Selenomonas* group.

INTRODUCTION

Kaolin is a rock consisting predominantly of the clay mineral kaolinite, which is important for the production of

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Abbreviations: AQDS, anthraquinone-2,6, disulfonate; NTA, nitroloacetic acid; OM, outer membrane; PCFO, poorly crystalline ferric oxide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Geobacter pickeringii* G13^T, *Geobacter argillaceus* G12^T and *Pelosinus fermentans* R7^T are DQ145535, DQ145534 and DQ145536, respectively.

paper as well as porcelain and ceramics. It is mined worldwide from subsurface kaolin lenses and has varying degrees of purity and whiteness. Kaolinite is a product of the weathering of aluminium silicate minerals, with feldspar being the most common source mineral. When conditions are favourable for weathering, white kaolin without discolouring impurities, such as iron oxides (goethite, hematite), sulfide (pyrite) and organic matter, is formed (Hurst & Pickering, 1997; Elzea Kogel *et al.*, 2002). Depending on their origin, kaolins and kaolin-containing source rocks are either mildly acidic (pH 5–5.5) (Elzea Kogel *et al.*, 2002) or slightly alkaline (pH 7–8.5) (Solodkii, 1995).

Subsurface kaolin lenses are not optimal environments for microbial growth due to the fine mineral particle size of clay and consequently the low hydraulic conductivity. However, cultivation-dependent most probable number analysis (Shelobolina *et al.*, 1999, 2005) and cultivation-independent lipid biomarker analysis (Osipov & Turova, 1997) have shown the presence of a variety of physiological groups of micro-organisms in kaolin samples of differing ages and origins collected from distant geographical locations in Russia, Ukraine and the USA. Stimulation of indigenous iron-reducing bacteria in kaolin results in a decrease of the Fe(III) : [Fe(II) + Fe(III)] ratio (Shelobolina *et al.*, 1999, 2005), iron solubilization (Turova *et al.*, 1996; Lee *et al.*, 1999; Shelobolina *et al.*, 1999) and in a dramatic increase in the efficacy of iron extraction by magnetic separation (Turova *et al.*, 1996; Vodyanitskii *et al.*, 1997; Shelobolina *et al.*, 1999). The ability of indigenous bacteria to influence iron mineralogy in kaolin suggests that bacteria have played a significant role in the natural removal of iron impurities from kaolin at the subsurface (Hurst & Pickering, 1997; Elzea Kogel *et al.*, 2002; Shelobolina *et al.*, 2005) and can be used for the development of microbially mediated industrial-scale iron removal from tinted kaolin (Avakyan *et al.*, 1997; Lee *et al.*, 1999). The goal of this project was to isolate representative Fe(III)-reducing bacteria from kaolin clays that might be involved in these processes. Three novel anaerobic Gram-negative micro-organisms were isolated from subsurface kaolin lenses in Russia and the USA. Two strains conserved energy from the oxidation of the organic matter with the reduction of Fe(III), whereas the third organism was capable of reducing Fe(III) only in the presence of a fermentable substrate.

METHODS

Study sites and sample collection. Strains G12^T and G13^T were isolated from subsurface kaolin lenses in Georgia (USA). Strain G12^T was isolated from tan kaolin of the Late Paleocene age from the Thiele Kaolin Co. Avant mine, 1 km west of GA highway 272, GA. Strain G13^T was isolated from kaolin from the Huber Formation of the Eocene age taken from the Thiele General Refractories clay mine, 3.2 km south of Tabernacle Church, GA. Both samples were collected in November 1999. Large pieces of kaolin were taken from freshly exposed surfaces in active mines using a mine excavator and/or a geological pickaxe. Samples were then wrapped with plastic and sent by an overnight delivery service to the laboratory. After arrival at the laboratory, all samples were placed into an N₂-filled glove bag. Large pieces of kaolin samples were broken with sterile knives. The middle portion of the samples was extracted, homogenized and dispensed into serum bottles (160 ml) for immediate experimentation or into large (2000 ml) sterilized Pyrex bottles with thick rubber stoppers for storage and/or later use.

Strain R7^T was isolated from primary kaolin of the Zhuravlinii Log eluvial deposit (located near the town of Plast, Chelabinsk region, Russia). Kaolin was collected in August 1995 using a mine excavator. The sample was placed in a large paper bag and shipped by regular mail to the State University of Management, Russia, to test its suitability for ceramics production. Immediately after arrival at the State University of Management, an aliquot of this kaolin was taken and placed into a

large (1000 ml) sterilized bottle with a thick rubber stopper. The bottle headspace was gassed with N₂. Microbial enrichments were established at the Institute of Microbiology of the Russian Academy of Sciences as part of a study into the role of micro-organisms within kaolins in the processes of iron solubilization and transformation (Shelobolina *et al.*, 1999).

Media and cultivation. Strict anaerobic techniques (Miller & Wolin, 1974; Balch *et al.*, 1979) were used throughout. An anaerobic basal bicarbonate-buffered freshwater (FW) medium (Lovley & Phillips, 1988) was dispensed into 27 ml anaerobic pressure tubes (Bellco Glass) or 160 ml serum bottles under N₂/CO₂ (80:20). The tubes or bottles were capped with butyl rubber stoppers and sterilized by autoclaving. The reducing agents were FeCl₂ (1.3 mM) for Fe(III)-containing media and cysteine (0.5 mM) for all other media and these were added after the medium was autoclaved.

Enrichment cultures with Georgia kaolin (USA) were initiated in 160 ml serum bottles containing 90 ml freshwater medium and 10 g clay from the site. Poorly crystalline ferric oxide (PCFO) (100 mmol l⁻¹) was prepared as described previously (Lovley & Phillips, 1986a) and used as the terminal electron acceptor for enrichment and isolation of the strains. Enrichments were established with hydrogen, acetate (10 mM), lactate (10 mM) or intrinsic organic matter (no electron donor added) as the electron donors. Strain G12^T was recovered from an enrichment culture established with intrinsic organic matter as the electron donor. The sample of tan kaolin from the Thiele Kaolin Co. Avant mine had 5.3% (w/w) of organic carbon. Acetate (10 mM) was the electron donor for the isolation of strain G12^T. Strain G13^T was isolated from an enrichment culture established with lactate (10 mM) as the electron donor. Isolates were recovered with a roll-tube method (Hungate, 1969). BBL agar (Becton Dickinson; 1.5%) was used as the solidifying agent. An inoculum (0.7 ml) from the 10-fold serial dilutions of the enrichment culture in a liquid FW medium was added to 27 ml pressure tubes containing 7 ml melted medium. The contents were mixed gently and the pressure tubes were rolled with a tube spinner (Bellco Glass). The roll-tubes were incubated vertically at 30 °C. Individual colonies were transferred to the pressure tubes with 2 ml liquid FW medium containing PCFO as the electron acceptor and either acetate (for strain G12^T) or lactate (for strain G13^T) as the electron donors.

The enrichment culture with Russian kaolin was initiated in 1995 and supported for 4 years in 27 ml pressure tubes containing 9 ml FW medium supplemented with 20 mM lactate and 100 mmol l⁻¹ PCFO. In 1999, the original enrichment culture was transferred to FW medium containing hydrogen as the electron donor, acetate as the carbon source and 100 mmol l⁻¹ PCFO as the electron acceptor. Strain R7^T was isolated from the resulting enrichment culture, which was transferred onto anaerobic agar plates solidified with 1.2% purified agar (BBL agar; Becton Dickinson) containing lactate (20 mM) as the electron donor and Fe(III)-pyrophosphate (10 mM) as the electron acceptor. Agar plates were incubated in an anaerobic chamber under an atmosphere of N₂/CO₂/H₂ (83:10:7) at 30 °C. For further study, individual colonies were transferred to liquid medium containing hydrogen as the electron donor, acetate as the carbon source and Fe(III)-pyrophosphate as the electron acceptor. Following isolation, strain R7^T was maintained on FW medium supplemented with 20 mM glucose.

Screening of potential electron donors and acceptors. The criterion used to determine whether isolates could utilize a particular electron donor or acceptor was the ability of the isolates to grow in presence of a potential donor or acceptor for 3–5 successive 10% transfers.

For strains G12^T and G13^T, the electron donor used to evaluate potential electron acceptors was acetate. Hydrogen was used for strain

R7^T with 5 mM acetate added as the carbon source. The following electron acceptors were tested: oxygen (5% of the headspace of the culture), PCFO (100 mmol l⁻¹), Fe(III) citrate (45 mM), Fe(III) nitriloacetic acid (NTA) (10 mM), Fe(III) pyrophosphate (10 mM), MnO₂ (30 mmol l⁻¹), nitrate (10 mM), nitrite (10 mM), thiosulfate (10 mM), sulfite (10 mM), sulfate (10 mM), fumarate (40 mM), anthraquinone-2,6-disulfonate (AQDS, 5 mM), S⁰ (1 g l⁻¹) and structural Fe(III) (6 mmol l⁻¹). Model ferruginous smectite (nontronite) SWa-1 from Grant County, WA, USA (Source Clays Repository, Clay Minerals Society) was used as a source of structural Fe(III). SWa-1 has a cation composition of Na_{0.87}(Si_{7.29}Al_{0.62})(Fe_{2.67}³⁺Fe_{0.01}²⁺Al_{1.08}Mg_{0.23}) (Maneau *et al.*, 2000). The clay suspension was prepared as described earlier (Shelobolina *et al.*, 2003). Fe(III) and Mn(IV) reduction were monitored as the accumulation of Fe(II) or Mn(II). AQDS reduction was monitored as a colour change in the medium from an opaque pink to a bright orange. Reduction of elemental sulfur was determined by adding elemental sulfur (1 g l⁻¹) to the medium and monitoring sulfide production by the methylene blue method (Cline, 1969). Growth on the other electron acceptors was monitored by measuring turbidity at 600 nm. Controls contained no electron acceptor.

The ability of washed cell suspensions to reduce U(VI) was determined as previously described (Shelobolina *et al.*, 2004) using uranyl acetate (1 mM) as the electron acceptor, acetate (5 mM) as the electron donor and the following reaction buffer (l⁻¹): 2.5 g NaHCO₃, 0.25 g NH₄Cl, 0.006 g NaH₂PO₄·H₂O, 0.1 g KCl. Cells were added to the reaction buffer to give a final protein concentration of 0.01 mg ml⁻¹.

Experiments to screen potential electron donors were performed in freshwater medium bubbled with N₂/CO₂ (80:20) and supplemented with either 100 mmol l⁻¹ PCFO (for strains G12^T and G13^T) or 10 mM Fe(III) NTA (for strain R7^T) as the electron acceptors. In order to decrease the amount of carry-over substrate, electron donor (acetate)-limited culture was used for the first transfer of strains G12^T and G13^T. Alternative electron donors were added to the medium from sterile anoxic stock solutions to give a final concentration of 10–20 mM, except for amino acids that were tested at a final concentration 0.1 g l⁻¹. Controls included no donor and donor alone (for potentially fermentable substrates).

Analytical techniques. Chelated and poorly crystalline forms of iron were quantified by the ferrozine assay (Lovley & Phillips, 1986b; Phillips & Lovley, 1987). Structural Fe(II) and Fe(III) in the clay were determined by hydrofluoric acid extraction followed by the 1,10-phenanthroline assay as described by Stucki (Stucki, 1981) and modified by Komadel and Stucki (Komadel & Stucki, 1988). Mn(II) concentrations were monitored as described previously (Kashefi & Lovley, 2000). Cells were counted with acridine orange staining and epifluorescence microscopy as described previously (Hobbie *et al.*, 1977).

16S rRNA gene sequencing and phylogenetic analysis. DNA was extracted from strains G12^T, G13^T and R7^T using the FastDNA SPIN kit (Bio101). The 16S rRNA gene was amplified using the bacterial forward primer 8F (AGAGTTTGATCMTGGCTCAG) and the universal reverse primer 1525R (AAGGAGGTFTCCARCC). These primers and the conserved internal primers 338F (AC-TCCCTACGGGAGGCAGC), 519F (CAGCACGCCGCGTAATFWC), 519R (GWATTACCGCGGCKGCTG), 907R (CCGTCAATTCMT-TTRAGTTT) and 1392R (ACGGGCGGTGTGTRC) were used to obtain the nearly complete sequence. PCR mixtures contained: a 1 × concentration of Q and *Taq* polymerase buffers (Qiagen), 0.5 µg bovine serum albumin, 200 µM deoxyribonucleoside triphosphates (Sigma-Aldrich), 25 pmol forward and reverse primers (Sigma) and 1.25 U *Taq* polymerase (Qiagen). PCR tubes containing all the reaction components except the template and *Taq* polymerase were UV irradiated for 7 min to ensure sterility. Reactions were performed in a Peltier thermal cycler (PTC 200) beginning with a 5 min denaturation

at 95 °C and then 25–30 cycles of 94 °C (30 s), 45 °C (1 min), 72 °C (1 min) and a final 10 min elongation at 72 °C. The 16S rRNA gene fragments from strains G12^T and G13^T and R7^T were compared with the GenBank nucleotide and protein databases using the BLASTN and BLASTX algorithms (Altschul *et al.*, 1990). Representative sequences from the *Sporomusa–Pectinatus–Selenomonas* group, members of the genus *Geobacter* and other genera were obtained from GenBank for phylogenetic analysis. Nucleotide sequences were manually aligned in the Genetic Computer Group (GCG) sequence editor (Wisconsin Package version 10). Aligned sequences were imported into PAUP 4.0b 4a (Swofford, 1998) where phylogenetic trees were inferred. Identical branching orders were observed with maximum-parsimony and distance-based algorithms when 16S rRNA gene sequences were compared. Bootstrap values were calculated by distance analysis.

Determination of the G+C content of DNA. The Deutsche Sammlung von Mikroorganismen und Zellkulturen identification service performed the DNA G+C content analysis for strain R7^T (Cashion *et al.*, 1977). The DNA G+C content of strains G12^T and G13^T was determined using a fluorometric technique employing real-time PCR (Gonzalez & Saiz-Jimenez, 2002, 2004). Genomic DNA standards from *Clostridium acetobutylicum* (ATCC 824D; 30 mol% G+C), *Escherichia coli* K-12 (ATCC 700926; 50 mol% G+C), *Pseudomonas aeruginosa* PAO1 (ATCC 47085D; 66 mol% G+C) and *Halobacterium* sp. (ATCC 700922D; 67 mol% G+C) were obtained from the ATCC. Thermal denaturation was performed with approximately 2.5 µg DNA from each novel isolate. Thermal conditions consisted of a ramp from 55 °C to 90 °C at 0.5 °C min⁻¹ on a Bio-Rad iCycler. Fluorescent melt curves for both sample and standard DNA were generated in triplicate. The DNA G+C content for strains G12^T and G13^T was calculated using linear regression analyses of melting temperatures (*T*_m) against the G+C content of the standard DNA.

RESULTS AND DISCUSSION

Characteristics of strains G12^T and G13^T

Cells of strains G12^T and G13^T were Gram-negative, motile, regular rods, 1–2 µm long and 0.6 µm in diameter. On anaerobic solidified FW medium, colonies were 1–2 mm in diameter. Colonies were pink on fumarate (20 mM, strain G13^T) due to the high *c*-type cytochrome content of cells of species of the genus *Geobacter* (Lovley *et al.*, 2004) and black on PCFO (100 mM, strains G12^T and G13^T) due to formation of dark coloured Fe(II)-bearing minerals such as magnetite (Lovley *et al.*, 1987). When grown with Fe(III) as the electron acceptor, cells had one lateral flagellum (Fig. 1a, b).

Cells of strain G13^T exhibited multiple short and elongated tubular projections (blebs) on the cell surface (Fig. 1b). Although blebbing of outer membrane (OM) followed by the formation of membrane vesicles has been described for a number of other Gram-negative bacteria (Beveridge, 1999; Kuehn & Kesty, 2005), this is the first *Geobacter* species for which formation of large quantities of blebs has been demonstrated. There are several hypotheses for the formation of blebs. Blebbing of the OM could be result of an overproduction of OM, compared with peptidoglycan (Wensink & Witholt, 1981) or the result of cell wall turnover during growth (Zhou *et al.*, 1998). Blebbing of the

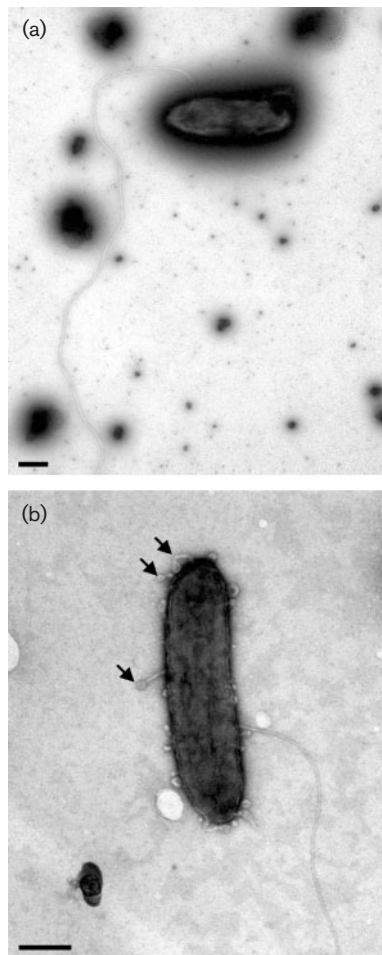


Fig. 1. Morphology of negatively stained cells of strains G12^T (a) and G13^T (b). Both strains were grown on acetate as the electron donor and PCFO as the electron acceptor. Cells were harvested in the middle exponential phase. Cells of strain G13^T exhibit short and elongated tubular projections (blebs) on the cell surface (b, shown with arrows). Bars, 0.5 μm.

OM increases the surface area of the cells, which might improve nutrient uptake. Formation of blebs in an Fe(III)-reducing bacterium, *Acidophilium cryptum* JF-5, has been hypothesized to enhance cellular contact with non-soluble Fe(III) hydroxides that are utilized as electron acceptors (Küsel *et al.*, 1999). In addition, when OM vesicles form as the result of OM blebbing, they entrap some of the underlying periplasm which enables the bacteria to secrete proteins, lipids and DNA into the extracellular medium (Forsberg *et al.*, 1981; Dorward *et al.*, 1989; Dorward & Garon, 1990; Kadurugamuwa & Beveridge, 1996; Kadurugamuwa *et al.*, 1998; Li *et al.*, 1998; Ciofu *et al.*, 2000; Kobayashi *et al.*, 2000; Yaron *et al.*, 2000). Vesicles can also mediate the coaggregation of bacteria, enabling biofilm formation and colonization (Grenier & Mayrand, 1987; Whitchurch *et al.*, 2002). Therefore, the formation of blebs may give strain G13^T a growth advantage over other micro-organisms.

With acetate (20 mM) as the electron donor and ferric citrate (50 mM) as the electron acceptor, growth of strains G12^T and G13^T was most rapid at 30 °C and there was no growth at temperatures below 10 °C. The upper growth temperature was 36 °C. Optimal pH for growth was 6.2–6.8 for strain G12^T and pH 6.6–7.2 for strain G13^T. No growth was observed at pH values lower than 5.8 for either isolate. The upper pH value for growth was 7.4 for strain G12^T and 8.0 for strain G13^T.

With acetate (20 mM) serving as the electron donor, both strains were capable of reducing the following electron acceptors: 60–100 mmol PCFO I⁻¹, ferric citrate (50 mM), Fe(III) NTA (10 mM), Fe(III) pyrophosphate (10 mM), 30 mmol MnOOH I⁻¹ and 1 g elemental sulfur I⁻¹ (Table 1). In addition, strain G13^T reduced a humic acid analogue, AQDS, fumarate (20 mM) and malate (20 mM). Strain G12^T also reduced nitrate (5 mM). Both strains were able to reduce U(VI) (1 mM) in cell suspension. The following electron acceptors were tested but not utilized by either strain: oxygen (5% of the headspace of the culture), sulfate (10 mM), sulfite (10 mM) and nitrite (5 mM). With PCFO as the electron acceptor, the isolates were capable of oxidizing the following electron donors (at concentrations 10–20 mM): ethanol, butanol, glycerol, acetate, lactate, butyrate, pyruvate and valerate. Strain G13 was also able to use methanol and succinate.

Table 1. Characteristics that differentiate strains G12^T and G13^T and related species of the genus *Geobacter*

Taxa: 1, *G. argillaceus* sp. nov. G12^T; 2, *G. pickeringii* sp. nov. G13^T; 3, *G. chapellei* 172^T (data from Coates *et al.*, 2001); 4, *G. hydrogenophilus* H-2^T (Coates *et al.*, 2001); 5, *G. metallireducens* GS-15^T (Lovley *et al.*, 1993); 6, *G. grbiciae* TACP-2^T (Coates *et al.*, 2001). +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
DNA G + C content (mol%)	58.0	61.4	50.2	58.4	56.6	57.4
Optimal temperature (°C)	30	30	25	35	30–35	30
Electron acceptors:						
AQDS	–	+	ND	ND	+	+
Elemental sulfur	+	+	+	–	–	ND
Fe(III) NTA	+	+	+	–	+	+
Ferric citrate	+	+	–	+	+	+
Fumarate	–	+	+	+	–	ND
Malate	ND	+	–	–	–	ND
MnOOH	+	+	–	–	+	ND
Nitrate	+	–	–	–	+	ND
Electron donors:						
Butyrate	+	+	–	+	+	+
Glycerol	+	+	ND	ND	–	ND
H ₂	–	–	–	+	–	+
Lactate	+	+	+	–	–	–
Methanol	–	+	–	–	–	–
Succinate	–	+	–	+	ND	–

The G+C content of the DNA was 58.0 mol% for strain G12^T and 61.4 mol% for strain G13^T.

Characteristics of strain R7^T

Cells of strain R7^T were motile, slightly curved rods, 0.6 × 2.0–6.0 μm (Fig. 2a). Spores were formed in the late exponential phase on all growth substrates. Spores were oval and always in a terminal position (Fig. 2b). The sporangia were distinctly swollen. The presence of up to six flagella was observed by electron microscopy. Flagella were peritrichous and up to 14 μm long and 15 nm wide. Ultrathin sections showed a Gram-negative type of cell wall, with the peptidoglycan layer and outer membrane clearly visible (Fig. 2b).

Optimal growth conditions for this strain were determined using FW medium supplemented with 20 mM glucose.

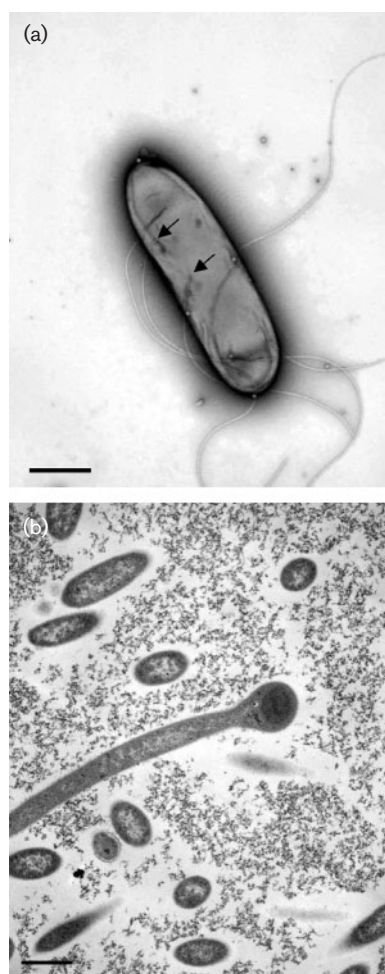


Fig. 2. Morphology of negatively stained (a) and ultrathin section (b) of cells of strain R7^T. Strain R7^T was grown on glucose. (a) General morphology of a cell showing peritrichous flagella insertion. Arrowheads indicate the flagella insertion points. Bar, 0.5 μm. (b) Ultrathin section of a cell of strain R7^T showing cell walls, membranes and spores. Bar, 1 μm.

Optimal growth was observed at 22–30 °C and no growth was observed at an initial temperature lower than 4 °C or higher than 36 °C. The pH range for growth was between 5.5 and 8, with an optimum at pH 7.

With hydrogen as the electron donor and acetate as the carbon source, strain R7^T was not capable of utilizing oxygen, AQDS, nitrate, sulfate, sulfite, thiosulfate, Fe(III) NTA, PCFO or elemental sulfur as the electron acceptor. Resting cells of strain R7^T did not reduce U(VI) when hydrogen was provided as the electron donor.

Although strain R7^T was enriched as a Fe(III) reducer, once isolated, this organism was capable of reducing Fe(III) only in the presence of a fermentable substrate. Strain R7^T could reduce ferric citrate (FC, Fig. 3a) or Fe(III) pyrophosphate (data not shown), but not Fe(III) NTA (Fig. 3a). The observed growth and reduction of Fe(III) when FC and Fe(III) pyrophosphate were provided as the electron acceptors can be explained by the presence of fermentable citrate and organic impurities in these substrates. Acetate was the major product of citrate fermentation by strain R7^T, with an acetate/citrate ratio of 1.9:1. This ratio decreased slightly in the presence of FC alone (1.7:1) or FC and hydrogen (1.4:1) (Fig. 3b). The presence of hydrogen did not increase the Fe(III) reduction rate (Fig. 3a) or cell yield

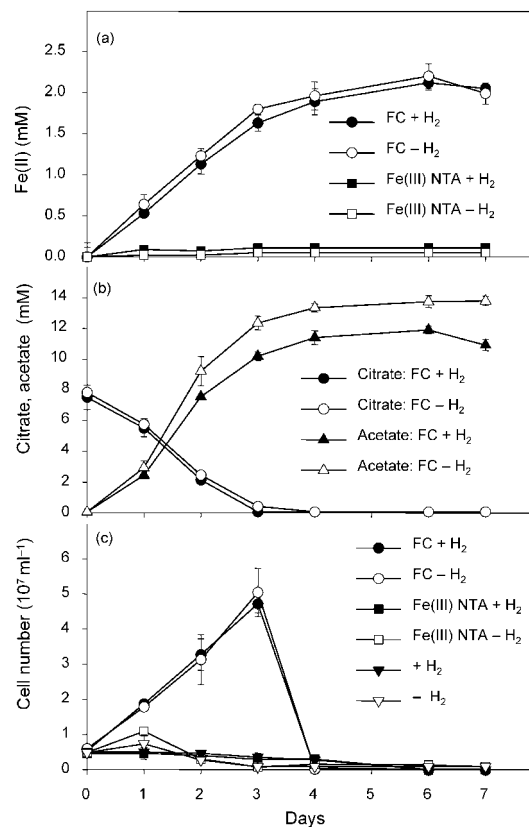


Fig. 3. Fe(III) reduction by strain R7^T. The results are the means of triplicate cultures. FC, ferric citrate.

(Fig. 3c), suggesting that strain R7^T could not use hydrogen as an electron donor. As follows from Fig. 3c, strain R7^T was not able to grow chemoautotrophically on H₂/CO₂. Strain R7^T reduced 0.3 mM Fe(III) per 1 mM citrate fermented. Together, these data suggest that strain R7^T, as with other fermentative bacteria (Dobbin *et al.*, 1999; Park *et al.*, 2001; Kim *et al.*, 2005; Bhushan *et al.*, 2006), utilizes Fe(III) as an electron sink. Strain R7^T was also capable of reducing small quantities (1–1.5 mM) AQDS in the presence of fermentable substrates, such as citrate or lactate (data not shown). The use of AQDS as a sink has been demonstrated for several fermentative bacteria, including species of the genera *Clostridium* (Bhushan *et al.*, 2006) and *Cellulomonas* (Borch *et al.*, 2005).

Strain R7^T fermented the following substrates: lactate, butyrate, pyruvate, malate, succinate, citrate, fumarate, fructose, glucose, mannitol, peptone, Casamino acids and yeast extract. The following substrates were tested but not utilized: glycerol, salicylic acid, ascorbate, 2,3-butanediol, maltose, gallic acid, pyrogallol, asparagines, histidine, glycine, leucine, lysine, serine, tyrosine, methionine, phenylalanine, proline, tryptophan, valine, glutamic acid and aspartic acid.

The G + C content of the DNA of strain R7^T was 41.0 mol%.

Phylogenetic analyses

BLAST and similarity analyses of 16S rRNA gene sequences indicated that strain G12^T was most similar to *Geobacter pelophilus* (sequence similarity 94.7%) and *Geobacter chapellei* (94.1%). Strain G13^T was most similar to *G. grbiciae* (95.3%) and *Geobacter metallireducens* (95.1%) in the class *Deltaproteobacteria*. Strain R7^T was most similar to the following micro-organisms of the *Sporomusa–Pectinatus–Selenomonas* phyletic group: '*Anaerospira hongkongensis*' (gene sequence similarity 90.2%), *Acetonema longum* (90.6%), *Dendrosporobacter quercicolus* (90.9%) and *Anaerosinus glycerini* (91.5%).

Phylogenetic analysis of these and other related sequences was performed using 1200 (Fig. 4a) and 1300 (Fig. 4b) bases for comparison. Placement of strains G12^T, G13^T and R7^T was consistent when using distance and maximum-parsimony analyses (Fig. 4a, b). The results indicated that strain G12^T was phylogenetically most closely related to *G. chapellei* and strain G13^T was most closely related to *Geobacter hydrogenophilus*, *G. metallireducens* and *G.*

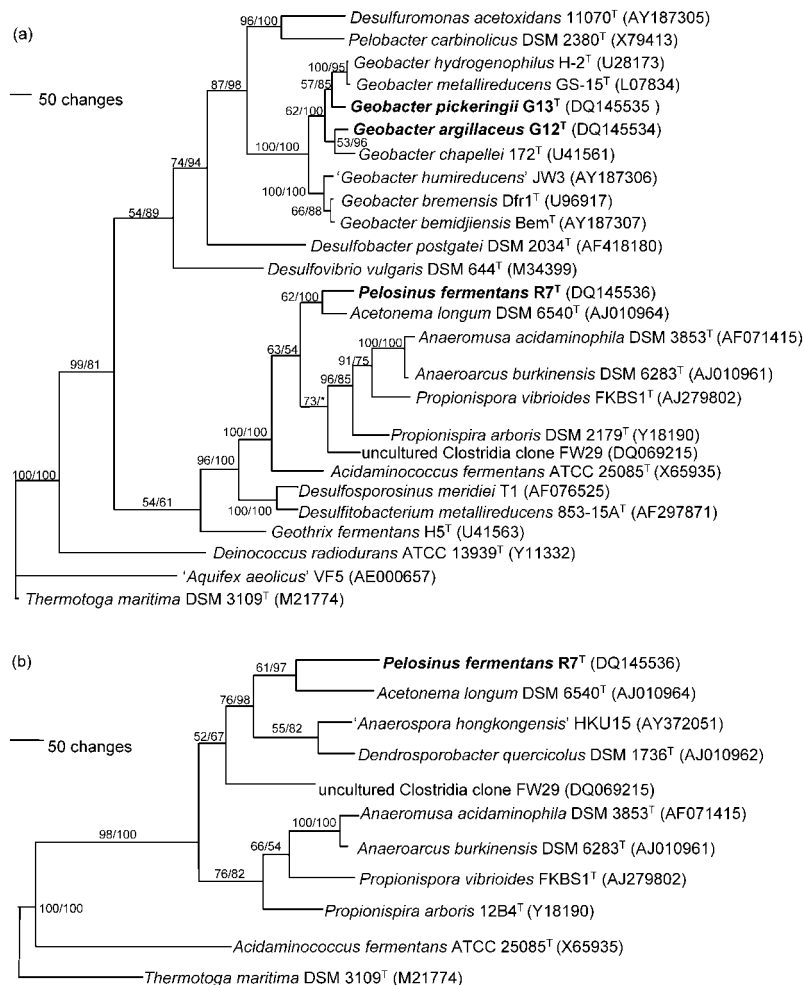


Fig. 4. Phylogenetic analysis of strains G12^T, G13^T and R7^T. (a) Phylogenetic tree comparing 16S rRNA gene sequences from the kaolin isolates. *Thermotoga maritima* and '*Aquifex aeolicus*' served as outgroups for the construction of the 16S rRNA gene tree. 1200 positions were considered. (b) Phylogenetic position of strain R7^T within the *Sporomusa–Pectinatus–Selenomonas* phyletic group. *Thermotoga maritima* served as an outgroup for the construction of this tree. 1300 positions were considered. Numbers next to branches indicate bootstrap values obtained from 100 replicates by maximum-parsimony/distance-based analysis. Asterisk indicates that distance analysis does not show separate branching for uncultured Clostridia clone FW29. Bars, 50 nucleotide changes.

grubicae (Fig. 4a). Based on the phenotypic differences between isolates G12^T and G13^T and closely related species of the genus *Geobacter* (Table 1), it is suggested that the isolates represent two novel species, *Geobacter argillaceus* sp. nov. (strain G12^T) and *Geobacter pickeringii* sp. nov. (strain G13^T).

Isolate R7^T grouped with members of the *Sporomusa*–*Pectinatus*–*Selenomonas* phyletic group of the phylum Firmicutes (Strömpl *et al.*, 1999), which is also referred to as clostridial cluster IX (Collins *et al.*, 1994) or the ‘*Sporomusa* branch’ (Biebl *et al.*, 2000) (Fig. 4a, b). This phyletic group contains a heterogeneous assemblage of organisms with Gram-negative cell walls, some of which are spore-forming. Strain R7^T was phylogenetically most closely related to *Acetonema longum* (Fig. 4b). Because of the low degree of similarity of the 16S rRNA gene sequence of strain R7^T with the closest members of the *Sporomusa*–*Pectinatus*–*Selenomonas* group, as well as significant phenotypic differences (Table 2), we propose to classify this strain as a representative of a new genus and species, *Pelosinus fermentans*.

Potential role of the novel isolates in iron cycling in kaolin strata

Of the three organisms described in this paper, two of the isolates are dissimilatory Fe(III)-reducing bacteria and belong to the genus *Geobacter*. Like other representatives of this genus (Lovley & Phillips, 1988; Caccavo *et al.*, 1994; Coates *et al.*, 1996; Straub *et al.*, 1998; Sung *et al.*, 2006), these isolates are capable of obtaining energy for growth by coupling the reduction of Fe(III) to complete the oxidation of acetate with the ratio of Fe(II) produced per acetate consumed close to 8 : 1. In contrast, strain R7^T represents a fermentative organism that is capable of reducing Fe(III) only in the presence of a fermentable substrate. Strain R7^T produced only 0.3 mM Fe(II) per 1 mM citrate consumed (Fig. 3). However, despite their lower potential for Fe(III) reduction, fermentative bacteria can be important to the iron geochemistry of kaolin due to the high organic matter content in kaolin (0.1–5.3 %, w/w). The organic matter of kaolins contains various potential electron donors and fermentable substrates for bacterial metabolism such as formate, acetate, lactate, propionate, butyrate, pyruvate,

Table 2. Characteristics that differentiate strain R7^T and related members of the *Sporomusa*–*Pectinatus*–*Selenomonas* group

Taxa: 1, *Pelosinus fermentans* sp. nov. R7^T; 2, *Acetonema longum* APO-1^T (data from Kane & Breznak, 1991); 3, *Dendrosporobacter quercicolus* DSM 1736^T (Stankewich *et al.*, 1971; Strömpl *et al.*, 2000); 4, *Anaerospinus glycerini* strain LGS 4^T (Schauder & Schink, 1989; Strömpl *et al.*, 1999); 5, ‘*Anaerospira hongkongensis*’ HKU15 (Woo *et al.*, 2005). ‘*Anaerospira hongkongensis*’ HKU15 was studied using ANI and ATB ID32A test kits (bioMérieux). The physiology of all other strains was examined using a classical cultivation approach. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5
DNA G + C content (mol%)	41.0	51.5	52.0–54.0	35.0	46.8
Cell shape	Slightly curved rods	Straight rods	Straight rods	Curved rods	Straight or slightly curved rods
Cell size (µm)	0.6 × 2–6	0.6 × 6–60	0.5 × 3	0.5 × 2–10	0.4–0.6 × 3.15–14.30
Temperature range (°C)	4–36	19–40	20–45	10–42	37
Temperature optimum (°C)	22–30	30–33	25–30	37	ND
pH range	5.5–8	6.4–8.6	ND	5–8.5	ND
pH optimum	7	7.8	ND	6.5–7.5	ND
Flagella	Up to 6 peritrichous	1–3 peritrichous	1–3 peritrichous	Not found	Polar flagellae or a tuft of flagellae inserted on one side
Spore formation	+	+	+	–	+
Growth on:					
Fructose	+	+	+	–	ND
Fumarate	+	+	ND	–	ND
Glucose	+	+	ND	–	–
Glycerol	–	–	+	+	–
H ₂ + CO ₂	–	+	–	–	ND
Lactate	+	–	ND	–	ND
Malate	+	–	ND	–	ND
Mannitol	+	+	–	–	–
Pyruvate	+	+	ND	–	ND
Succinate	+	–	ND	–	ND

oxalate and citrate (Shelobolina *et al.*, 1999, 2005) as well as humic and fulvic acids (Elzea Kogel *et al.*, 2002). At the same time, kaolins are characterized by low amounts of 'bioavailable' Fe(III) (0.5 M HCl extractable hydroxylamine reducible; 0.0–0.9 mmol kg⁻¹) compared with total Fe(III) (hydrofluoric acid-extractable, 7.7–48.6 mmol kg⁻¹) (Shelobolina *et al.*, 2005). Electron shuttling via humic acids can explain high levels of microbial Fe(III) reduction in kaolins in model experiments despite their low 'bioavailable' Fe(III) content (Shelobolina *et al.*, 2005). The important role of fermentative bacteria in humic acid-mediated Fe(III) reduction has been demonstrated for freshwater lake sediments (Kappler *et al.*, 2004). Together, these data suggest that both dissimilatory Fe(III)-reducing and fermentative bacteria could be important contributors to microbial Fe(III) reduction in kaolin and can be used as model organisms to study biotransformations of iron minerals in kaolin.

Description of *Geobacter argillaceus* sp. nov.

Geobacter argillaceus (ar.gil.la'ceus. L. masc. adj. *argillaceus* of clay).

Cells are Gram-negative, motile, regular rods, 1–2 µm long and 0.6 µm in diameter. Cells have one lateral flagellum. Optimal growth occurs at pH 6.2 to 6.8 and no growth occurs at initial pH values lower than 5.8 or higher than 7.4. Growth is most rapid at 30 °C; no growth occurs at temperatures below 10 °C or above 36 °C. Uses PCFO, ferric citrate, Fe(III) NCA, Fe(III) pyrophosphate, MnOOH, elemental sulfur and nitrate as electron acceptors. Reduces U(VI) in cell suspension. Oxidizes the following electron donors: ethanol, butanol, glycerol, acetate, lactate, butyrate, pyruvate and valerate. The G + C content of the DNA of the type strain is 58.0 mol%.

The type strain, G12^T (= ATCC BAA-1139^T = JCM 12999^T), was isolated from subsurface kaolin strata in Georgia, USA.

Description of *Geobacter pickeringii* sp. nov.

Geobacter pickeringii (pic.ke.rin'gii. N.L. gen. masc. n. *pickeringii* of Pickering, named in honour of Sam M. Pickering Jr., for his significant contributions to the study of Georgia kaolins).

Cells are Gram-negative, motile, regular rods, 1–2 µm long and 0.6 µm in diameter. Cells have one lateral flagellum and exhibit numerous short tubular projections on the cell surface. Optimal growth occurs at pH 6.6 to 7.2; no growth occurs at initial pH values lower than 5.8 or higher than 8.0. Growth is most rapid at 30 °C; no growth occurs at temperatures below 10 °C or above 36 °C. Uses PCFO, ferric citrate, Fe(III) NCA, Fe(III) pyrophosphate, MnOOH, elemental sulfur, AQDS, fumarate and malate as electron acceptors. Reduces U(VI) in cell suspension. Oxidizes the following electron donors: methanol, ethanol, butanol, glycerol, acetate, lactate, butyrate, pyruvate, succinate and

valerate. The G + C content of the DNA of the type strain is 61.4 mol%.

The type strain, G13^T (= ATCC BAA-1140^T = DSM 17153^T = JCM 13000^T), was isolated from sedimentary kaolin strata in Georgia, USA.

Description of *Pelosinus* gen. nov.

Pelosinus (Pe.lo.si'nus. Gr. masc. n. *pelos* mud or clay; L. masc. n. *sinus* bend; N.L. masc. n. *Pelosinus* a curved organism from clay).

Cells are Gram-negative, motile, slightly curved rods. Spore-forming. Sporangia are distinctly swollen. Obligately anaerobic. Mesophilic. Does not produce acetate from H₂ + CO₂. Fermentative. Uses a variety of fermentative substrates, but does not ferment glycerol. The type species is *Pelosinus fermentans*.

Description of *Pelosinus fermentans* sp. nov.

Pelosinus fermentans (fer.men'tans. L. part. adj. *fermentans* fermenting).

Displays the following features in addition to those given in the genus description. Cells are 2–6 µm long and 0.6 µm in diameter. Cells have up to 6 peritrichous flagella. Oval endospores are formed terminally. Optimal growth occurs at pH 7.0; no growth occurs at initial pH values lower than 5.5 or higher than 8.0. Growth is most rapid at 22–30 °C; no growth occurs at temperatures below 4 °C or above 36 °C. Does not respire anaerobically with Fe(III), AQDS, nitrate, sulfate, sulfite, thiosulfate or elemental sulfur, but is capable of using Fe(III) and AQDS as an electron sink in the presence of fermentable substrate. Does not reduce U(VI) in cell suspension. Ferments the following substrates: lactate, butyrate, pyruvate, malate, succinate, citrate, fumarate, fructose, glucose, mannitol, peptone, Casamino acids and yeast extract. The G + C content of DNA of the type strain is 41.0 mol%.

The type strain, R7^T (= DSM 17108^T = ATCC BAA-1133^T), was isolated from subsurface primary kaolin deposits in Russia.

ACKNOWLEDGEMENTS

We would like to thank E. L. Blunt for her technical assistance. We thank S. M. Pickering and J. Elzea Kogel for advice and help with collecting GA kaolins and Thiele Kaolin Co., GA, for access to its mines. We also thank R. A. Khalilullova and Yu. T. Platov for providing us with a sample of kaolin from the Zhuravlinii Log deposit, Plast, Chelabinsk region, Russia. We thank L. Yin at the University of Massachusetts, USA, for helping with the electron micrographs. We are grateful to two anonymous reviewers and to the editor, Professor Peter Kämpfer, for useful comments. This work was supported by NSF NATO (grant DGE-9902554) and by Department of Energy (grant DE-FG02-97ER62475).

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